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GRAM-NEGATIVE BACTERIA:

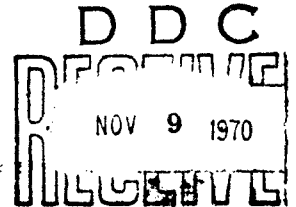
CHARACTERIZATION OF THE PROTEIN MOIETY ISOLATED BY PHENOL TREATMENT

OF ENDOTOXIN FROM *S. MARCESCENS* 08 AND *E. COLI* 0 141:K85 (B)

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Technical Report No. 28

University of Oklahoma Medical Center THEMIS Contract



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# Abstract

The so-called "simple" proteins isolated from the endotoxin of S. marcescens 08 and whole cells of E. coli 0 141:K85(B) by aqueous phenol treatment were characterized by the determination of hydrodynamic properties, electrophoretic behavior, immunochemical specificity and chemical analysis. The chemical composition of both proteins revealed the presence of lipid A constituents such as glucosamine, phosphorus and fatty acids; the  $\beta$ -hydroxy myristic acid regarded as the characteristic marker for lipid A was present in all protein preparations. Evidence presented in this paper indicates that a portion of lipid A is firmly bound to the protein moieties. Therefore, endotoxin protein preparations isolated by aqueous phenol cannot be classified as "simple" proteins if this term implies absence of compounds other than amino acids. The protein moieties from S. marcescens and E. coli were immunogenic and possessed a common antigenic determinant.

It is generally accepted that the endotoxin or somatic O-antigen complexes occurring in the cell walls of Gram-negative bacteria represent macromolecular entities composed of lipid, polysaccharide and protein moieties (1, 2). Evidence for this structural complexity was presented in early studies by Morgan and Partridge (3-5) who showed that endotoxins isolated from Shigella dysenteria and Salmonella typhosa could be resolved by successive treatments with formamide and 90% phenol into a loosely bound lipid (lipid B), "undegraded" polysaccharide (lipopolysaccharide consisting of a polysaccharide moiety and firmly bound lipid A), and a "simple" protein. Alternatively, the endotoxin could be dissociated by treatment with 1% acetic acid into a mixture of lipid B, "degraded" polysaccharide and "conjugated" protein. The latter fragment could be converted into "simple" protein either by the action of 90% phenol or by repeated treatments with 0.1 N sodium hydroxide (4). Subsequently, several investigators have confirmed the lipopolysaccharide-protein

nature of intact endotoxins isolated either by the extraction of whole cells with various organic solvents (3-10), trichloroacetic acid (9, 11-13), or by precipitation from culture fluids (14-16).

It has been suggested that, except for decreased water solubility (4) and reduced immunogenicity (1, 2), the removal of the protein moiety by chemical (8, 13, 14, 17) or enzymatic (18, 19) treatment of intact endotoxin has little, if any, effect on the retention of characteristic serological and toxic properties of the remaining lipopolysaccharide fragments. The relative lack of interest in studying either the properties or mode of linkage of the protein moiety in intact endotoxin may be due to the generally accepted assumption that the protein functions primarily as an inert "carrier" of other biologically active components.

There are, however, several as yet inadequately explained observations that seem to justify a reevaluation of the structural,

biological and morphological roles of the protein moiety as an integral part of the intact endotoxin. The variable amounts of bound amino acids (1, 8, 9, 12-14, 18-20) found in "deproteinized" lipopolysaccharide preparations may represent the linking fragments of the protein moiety. Although the site and nature of the protein linkage in intact endotoxin is unknown, it has been suggested (1) that the protein moiety may be bound to lipid A through an aminodicarboxylic acid; indeed, glutamic acid and aspartic acid have been repeatedly detected as the major amino acids in lipid A preparations (1, 20-23). Binkley et al., (7) have also suggested a possible close relationship between the protein and lipid moieties by demonstrating that the toxicity of the endotoxin of Shigella flexneri resides either in the protein or in the polysaccharide moiety depending upon the mode of its dissociation. Partial hydrolysis of the endotoxin with picric acid yielded a "toxic" protein which could be converted by treatment with alkaline alcohol into a non-toxic "simple" protein;

the "toxic" protein differed from the "simple" protein also by its significantly higher phosphorus and lower nitrogen content. It has already been proposed (2) that the "toxic" protein consists most probably of an alkali sensitive lipid A-protein complex. However, direct chemical proof for the existence of such a complex is still lacking.

This report represents the initial phase of a study of the structural and functional roles of endotoxin protein. It describes the physical, chemical and immunological properties of the protein moieties isolated by phenol treatment of endotoxins from Serratia marcescens 08 and Escherichia coli 0 141:K85(B).

#### MATERIALS AND METHODS

##### Bacteria

Cells of the chromogenic strain Serratia marcescens 08, cultivated and harvested as described previously (24), were supplied by General Biochemicals, Chagrin Falls, Ohio.

The dialyzed and lyophilized phenol phase of Escherichia

coli O 141:K85(B) was supplied by Dr. K. Jann, Max-Planck

Institut für Immunobiologie, Freiburg, Germany.

Extraction of the Cells of *S. marcescens* 08

Wet cells washed with distilled water were extracted twice with 5% trichloroacetic acid (100 g cells/200 ml trichloroacetic acid) according to a modified method of Boivin et al., (25).

The combined extracts were dialyzed against distilled water for 48 hours, concentrated by vacuum distillation to approximately 50 ml, and centrifuged in a Spinco Model L ultracentrifuge for one hour at 40,000 rpm (105,000 x g). The lyophilized nucleic acid-free sediment (whole endotoxin, LPS-U-S) was treated with 45% aqueous phenol for 30 minutes at 68° according to the method of Westphal et al., (17). The cooled water and phenol phases were separated, and the phenol phase was used for the isolation of the protein moiety.

Extraction of the Cells of *E. coli* O 141:K85(B)

Twenty grams of dry cells were treated with 45% aqueous



phenol for 10-15 minutes at 68° without previous extraction with trichloroacetic acid. After cooling, the phenol and water phases were separated by centrifugation. Addition of 3-4 volumes of methanol to the phenol phase resulted in the formation of a precipitate which was removed by centrifugation, resuspended in distilled water, dialyzed and lyophilized. To isolate the protein moieties of S. marcescens and E. coli under identical experimental conditions, 4-5 g of E. coli precipitate were dissolved in 500 ml 90% aqueous phenol by stirring for 12 hours at 35°.

Isolation of "Simple" Proteins (PX-S and PX-E) from

Phenol Phases of S. marcescens and E. coli

The phenol phases of S. marcescens and E. coli were washed several times with distilled water to remove the trace amounts of lipopolysaccharides and peptides (Fig. 1). The combined washings were lyophilized and tested immunologically for the possible presence of lipopolysaccharides and by high-voltage

paper electrophoresis for the presence of peptides. The disappearance of immunoprecipitin lines of lipopolysaccharides and the absence of peptide spots were used as the criteria for purity of the phenol phases. The addition of 9.5 volumes of 95% ethanol to the phenol phases and storage of these mixtures at  $-10^{\circ}$  for 7-10 days yielded a dark purple precipitate from S. marcescens and a white precipitate from E. coli. Precipitates were removed by low-speed centrifugation and washed twice with ethanol and several times with water. After lyophilization, these precipitates represented fractions P-S and P-E, respectively.

To remove any "free" lipids, fractions P-S and P-E were extracted with chloroform-methanol (2:1, v/v) in a Soxhlet extractor for 12 hours. The residual fractions, PX-S from S. marcescens and PX-E from E. coli represented the "simple" protein preparations. To detect any possible peptide or amino acid impurities, PX-S and PX-E were submitted to high-voltage paper electrophoresis (3000 V, 30 minutes, pyridine-acetate

buffer, pH 3.6, ninhydrin stain; Savant Instruments, Inc., Hicksville, New York). If ninhydrin-positive spots were detected, washing of PX-S and/or PX-E with distilled water was continued for several days until last traces of impurities were removed. Only such highly purified preparations of "simple" protein were used for characterization studies.

#### Oxidation of PX-S and PX-E

Fifty milligrams of PX-S or PX-E were oxidized by stirring with 25 ml freshly prepared performic acid for 24 hours at 0° according to a slightly modified method of Mueller et al., (26). The reaction mixture was either lyophilized or evaporated in a vacuum desiccator over KOH, and the residue was dissolved in distilled water. The aqueous solution was concentrated to dryness in a rotary evaporator at 36°. The slightly yellow colored residue was either dissolved in a boric acid buffer (50 ml of a mixture 0.1 M  $H_3BO_3/KCl$  + 20.8 ml of 0.1 M NaOH, diluted to 100 ml with distilled water, pH 8.9) or suspended in distilled

water, dialyzed for 7 days against distilled water at 4° and lyophilized. The oxidized "simple" proteins were designated as PXOD-S and PXOD-E, respectively.

#### Electrophoresis

Agarose electrophoresis was carried out in 1% agarose according to the procedure by Grabar and Williams (27) employing Veronal buffer, pH 8.6, ionic strength 0.05, or 0.05 M Tris-HCl buffer containing 0.5% SDS, pH 7.6. Samples were dissolved in 0.05 M Tris-HCl buffer containing 0.5% SDS, pH 8.6. The plates were fixed in a solution of acetic acid-ethanol-water (5:70:25, v/v), washed in distilled water and dried at room temperature. Amido Black 10B was used for protein staining.

Preparative agarose electrophoresis of PX-S was carried out on a "Fractophoretor" (Buchler Instruments, Fort Lee, New Jersey). Ten to twenty milligrams of PX-S dissolved in 1 ml 0.05 M Tris-HCl buffer containing 0.5% SDS, pH 7.6, were mixed with 0.5 ml 2% agarose in Veronal buffer, pH 8.6, ionic strength 0.1, at 85°.

This stacking gel was poured on top of a column (10 x 1.3 cm) of separating gel of 2% agarose in Veronal buffer. Electrophoresis was carried out with Veronal buffer, pH 8.7, ionic strength 0.1, at 25 mA. The 1-ml fractions were monitored by measuring absorption at 280 m $\mu$  and 470 m $\mu$ . The main fraction was dialyzed against distilled water and lyophilized.

Polyacrylamide gel electrophoresis was carried out with a Canalco Model 6 unit (Canal Industrial Corp., Bethesda, Md.) in 7.5% polyacrylamide gel employing a continuous buffer system of Tris-glycine, pH 8.8, at 5 mA per tube, according to the method of Davis (28).

#### Analytical Ultracentrifugation

Ultracentrifugal analyses were carried out in a Spinco Model E ultracentrifuge equipped with a phase plate schlieren diaphragm and an automatic temperature control unit. Sedimentation coefficients were determined at constant temperature (25-26°) employing rotor speeds of 56,100 rpm. The apparent diffusion

coefficients of PXOD-S and PXOD-E were determined at rotor speed of 12,590 rpm by applying the height-area method.

#### Infrared Spectroscopy

Infrared spectra were obtained with a Beckman Infrared Spectrophotometer JR10 using potassium bromide pellets (1.5-1.8 mg of substance and 250 mg KBr).

#### Paper and Thin-layer Chromatography

Carbohydrates were separated on Whatman No. 1 paper according to the procedure by Colombo et al., (30). The solvent systems used were a) ethyl acetate-pyridine-water (3.5:1:1.5, v/v) or b) n-butanol-pyridine-water (1092:728:546, v/v). The spots were detected by spraying with alkaline silver nitrate or ninhydrin, and identified by comparison with known standards.

Lipids were separated by thin-layer chromatography according to the method of Mangold et al., (31). Glycerides, fatty acids and phospholipids were detected by spraying plates with 50% sulfuric acid and subsequent charring or with bromthymol blue (32)

phospholipids were detected also by spraying with molybdenum blue (33) and ninhydrin.

#### Elementary Analyses

Samples were dried in high vacuum over  $P_2O_5$  for 24-48 hours. The analyses for carbon, hydrogen, nitrogen and ash were performed by Galbraith Laboratories, Knoxville, Tennessee. Phosphorus was determined according to the method of Gerlach and Deuticke (34).

#### Amino Acid Analyses

Duplicate samples of protein preparations (2 mg) were hydrolyzed in 5.7 N HCl (1 ml for each mg of protein) in evacuated, sealed tubes at 110° for 24 and 72 hours. To each sample 0.05  $\mu$ M of norleucine and 56.8  $\mu$ g of heptadecanoic acid (Supelco, Inc., Bellefonte, Pennsylvania) were added as internal standards. The hydrolysates were extracted three times with chloroform to remove fatty acids. The chloroform extracts were washed three times with distilled water to remove traces of amino

acids. The amino acid hydrolysates and washings were evaporated to dryness in vacuum and the residues dissolved in 2 ml of 0.2 N Na-citrate buffer, pH 2.2. Analysis was performed on a Beckman Model 120C amino acid analyzer according to an accelerated automatic procedure on spherical resins (35). Methionine and half-cystine were determined as methionine sulfoxide and cysteic acid after oxidizing protein samples with performic acid (36). Tryptophan was determined according to the method of Spies and Chambers (37). Values for serine, threonine and tyrosine were obtained by linear extrapolation of average recoveries from 24 and 72 hours of hydrolysis. Values for valine and isoleucine represent average recoveries obtained after 72-hour hydrolysis.

#### Determination of Fatty Acids

Chloroform extracts of protein hydrolysates containing heptadecanoic acid as internal standard were evaporated to dryness in high vacuum, and the residues were dissolved in 0.5 N methanolic KOH and boiled under reflux for 1 hour. Unsaponifiable matter



was extracted with n-heptane. After acidification the fatty acids were extracted with chloroform and esterified by boron trifluoride methanol reagent (Applied Science Laboratories, Inc., State College, Pennsylvania). The fatty acid methyl esters were analyzed qualitatively and quantitatively by gas-liquid chromatography on a Barber-Colman gas chromatograph, Series 5000, equipped with a flame detector. The glass column was packed with 15% diethyl glycol succinate on chromosorb W, 80-100 mesh (Applied Science Laboratories, Inc.). The chromatography was carried out at 172-175°.

The hydrogenation of the fatty acid methyl esters was carried out in a Brown Hydro Analyzer (38) (Delmar Scientific Laboratories, Inc., Maywood, Illinois). Acetylation of fatty acid methyl esters by freshly distilled acetic acid anhydride in distilled pyridine was used to convert hydroxy fatty acids into acetoxy fatty acids (39). Fatty acid methyl ester standards were obtained from Applied Science Laboratories, Inc. A sample

of  $\beta$ -hydroxy myristic acid was kindly supplied by Professor J. Asselineau, Faculté de Sciences, Toulouse, France.

#### Determination of Carbohydrates

Anthrone positive carbohydrates were determined according to the method by Koehler (40).

Glucosamine was determined by the method of Rondle and Morgan (41) and by the amino acid analyzer. Protein samples were hydrolyzed with 5.7 N HCl in evacuated, sealed tubes at 110° for 16, 24 and 72 hours, and the obtained values for glucosamine were extrapolated to zero time.

#### Immunological Methods

The immunological properties of protein preparations were studied by double diffusion (29) and immunoelectrophoresis (27) in 1% agar gel employing Veronal buffer, pH 8.6, ionic strength 0.1. Plates allowed to develop for 24-30 hours were washed several times with 0.15 M NaCl and distilled water and dried at room temperature. They were stained for protein with Amido Black 10B.

Antigens and corresponding antisera were labeled with the same letters; the antigens were marked with capital and the antisera with small letters. Antisera were prepared against following preparations:

<u>Antigens</u>		<u>Antisera</u>	
PX-S	A	Anti PX-S	a
PX-E	B	-	-
PXOD-S	C	Anti PXOD-S	c
PXOD-E	D	Anti PXOD-E	d
LPS-U-S	K	Anti LPS-U-S	k

To isolate the polysaccharide side chain (L), intact endotoxin (LPS-U-S) from S. marcescens 08 was hydrolyzed with 1% acetic acid for 4 hours at 90°. The hydrolysate was centrifuged at 10,000 rpm for 20 minutes and the chloroform-extracted supernate was dialyzed against distilled water. Lyophilization of inner dialyzate yielded an immunochemically-homogeneous side chain. An antiserum was prepared also against whole cells of S. marcescens 08.

White rabbits were immunized by three successive intraperitoneal injections of a mixture consisting of 1 mg of each antigen dissolved in 2 ml 0.05 M Tris-HCl buffer containing 0.5% SDS and emulsified with 2 ml of Freund's complete adjuvant. The injection.

were administered in weekly intervals. Identical antisera were also obtained when PX-S, PXOD-S and PXOD-E were suspended in 0.9% NaCl. Blood was obtained by cardiac puncture. Antibodies were first demonstrated after the second injection. The Tris-HCl-SDS buffer did not induce formation of antibodies.

## RESULTS

### Isolation of "Simple" Proteins

To isolate "simple" protein, the trichloroacetic acid extracted endotoxin complex of S. marcescens 08 was treated with aqueous phenol. The purple colored fraction (P-S) isolated from phenol phase by precipitation with ethanol (Fig. 1) contained about 20% lipids extractable by chloroform-methanol. These lipids were identified by thin-layer chromatography as unesterified fatty acids, glycerides and phospholipids; triglycerides represented the major glyceride component and phosphatidyl ethanolamine the principal phospholipid. Only a relatively small amount of pigment, prodigiosin or its derivatives, could be extracted from P-S. After

removal of fraction P-S, addition of ethanol and prolonged storage of the remaining mixture at  $-10^{\circ}$  resulted in no further precipitation of protein. Phenol was removed from the filtrate by steam distillation and dialysis against distilled water. After lyophilization, the residue, representing less than 10% of the material originally present in the phenol phase, consisted of pigments soluble in several organic solvents. Thus, the phenol phase of S. marcescens 08 contained approximately 70% "simple" protein (PX-S), 20% "free" lipids and 10% "free" pigments.

Since it was proposed without rigorous experimental evidence that the protein moieties of endotoxins isolated from Gram-negative bacteria might be similar (2), the "simple" protein (PX-E) was isolated also from a strain of E. coli according to the procedure outlined in Fig. 1; the phenol phase was obtained by direct extraction of cells rather than by treatment of the endotoxin complex. The lyophilized fraction P-E was extracted with chloroform-methanol and, similarly to fraction P-S, contained

approximately 20% "free" lipids. However, in contrast to PX-S, the "simple" protein (PX-E) accounted for only 40-50% of the material originally dissolved in phenol.

#### Physical-chemical Properties of "Simple" Proteins

The "simple" proteins, PX-S and PX-E, were insoluble in water, various buffer systems, dilute acid and alkali, and in most organic solvents. Both protein preparations were soluble in dimethylsulfoxide; they were solubilized also in 0.05 M Tris-HCl buffer, pH 8.6, containing 0.1-0.2% SDS. PX-S dissolved in Tris-HCl buffer exhibited a single, symmetrical peak in the analytical ultracentrifuge (Fig. 2) with  $s_{\text{obs}} = 2.4S$ .

On agarose gel electrophoresis both PX-S and PX-E migrated as single bands (Fig. 3, patterns a and b). Preparative agarose gel electrophoresis of PX-S yielded a single fraction characterized by a symmetrical elution curve with absorption maxima at 280 m $\mu$  and 470 m $\mu$  (absorption of prodigiosin). PX-S showed a single band also on polyacrylamide gel electrophoresis (Fig. 4).

Since the purple-colored band of PX-S was identical with the band obtained upon staining with protein stain, it was concluded that prodigiosin or one of its derivatives was bound to PX-S.

There was a striking similarity between the infrared spectra of PX-S and PX-E (Fig. 5). In addition to the broad absorption at  $3400-3280\text{ cm}^{-1}$  ( $\nu$  OH ass. or  $\nu$  NH ass.) and  $2960-2850\text{ cm}^{-1}$  ( $\nu$   $\text{CH}_3 + \text{CH}_2$ ) and the amide I and amide II bands at  $1650\text{ cm}^{-1}$  and  $1520\text{ cm}^{-1}$ , there were also bands at  $1230\text{ cm}^{-1}$  and  $1060\text{ cm}^{-1}$ . The former absorption could possibly be assigned to  $\nu$  P = O ass., the band at  $1060\text{ cm}^{-1}$  may be due to  $\delta$  OH and  $\nu$  C-O. The chemical analysis of PX-S and PX-E supports this interpretation. That a small shoulder on the amide I absorption band at  $1740\text{ cm}^{-1}$  may have been caused by  $\nu$  C=O ester absorption was confirmed later by other means.

#### Oxidation of "Simple" Proteins PX-S and PX-E

To obtain soluble products, PX-S and PX-E were oxidized by performic acid; a prolonged treatment for 24 hours was essential.

for the oxidative degradation of the pigment of PX-S. The dialyzed and lyophilized oxidation products PXOD-S and PXOD-E were obtained in a 90% yield. Both protein preparations were readily soluble in borate buffer, pH 8.9, and exhibited single, symmetrical peaks in the analytical ultracentrifuge (Figs. 6 and 7). PXOD-S was characterized by  $s_{\text{obs}} = 1.22\text{S}$ , and  $D_{\text{app}} = 16.2 \times 10^{-7} \text{cm}^2 \text{sec}^{-1}$ . corresponding values for PXOD-E were 1.14S and 9.7.

PXOD-S and PXOD-E migrated on agarose electrophoresis as single bands of approximately the same mobility (Fig. 3, patterns c and d). The oxidized products migrated faster and showed a weaker reaction with Amido Black 10B than the corresponding parent proteins. The infrared spectra of PXOD-S and PXOD-E were essentially the same as those of PX-S and PX-E (Fig. 5).

#### Chemical Characterization of Protein Preparations

The results of the elementary analysis and chemical composition of PX-S, PX-E, PXOD-S and PXOD-E are shown in Table 1. Hydrolysates of nonoxidized and oxidized forms of "simple" proteins



consisted not only of amino acids, but also of glucosamine, phosphate and fatty acids. PX-E contained slightly more nitrogen and sulfur but less fatty acids, glucosamine and phosphate than PX-S. Although analysis indicated the presence of a very small amount of anthrone-positive carbohydrate, no sugars other than glucosamine could be detected by paper or thin-layer chromatography.

It was shown by gas liquid chromatography (Table 2) that both "simple" proteins contained  $\beta$ -hydroxy myristic acid, recognized as the characteristic marker of lipid A (1, 2, 42). Beta-hydroxy myristic acid was the principal fatty acid of PX-S, whereas stearic acid was the major fatty acid of PX-E. The distribution of fatty acids in PX-S was similar to that of lipid A isolated from the lipopolysaccharide of S. marcescens 08 (24).

Amino acid compositions are shown in Table 3. Both PX-S and PX-E had relatively high contents of acidic amino acids, glycine and alanine. Half cystine was absent or found only in trace amounts.

The relative content of carbon, nitrogen, phosphate, glucosamine, amino acids and fatty acids of PXOD-S and PXOD-E was lower than that of PX-S and PX-E (Table 1). Prolonged oxidation of PX-S and PX-E by performic acid resulted also in partial hydrolysis of protein preparations. This hydrolytic effect of performic acid was clearly reflected in a changed amino acid composition (Table 3) and fatty acid distribution (Table 2) of PXOD-S and PXOD-E. The cleaved reaction products were identified as glucosamine, a phosphorus-containing compound (most probably a glucosamine phosphate), some amino acids and approximately 30-40% of the total fatty acids. The most significant change in the fatty acid distribution was an increased relative content of  $\beta$ -hydroxy myristic acid of both PXOD-S and PXOD-E. Mild alkaline hydrolysis of PX-S and PX-E with 0.5 N methanolic KOH resulted also in cleavage of 40-50% of total fatty acids and an increase in the content of  $\beta$ -hydroxy myristic acid. These results suggested that the hydrolytic action of performic acid affected the ester

linked fatty acids primarily. The remaining fatty acids were cleaved only by strong acid hydrolysis, and therefore were probably bound in PXOD-S and PXOD-E through amide linkages. Although the oxidized forms of "simple" proteins represent somewhat artificial products, their solubility properties could be utilized advantageously for physical-chemical and immunological studies and characterization.

#### Immunological Properties of Protein Preparations

The "simple" protein PX-S(A) and the oxidized preparations PXOD-S(C) and PXOD-E(D) were found to be immunogenic in rabbits. Because of poor solubility in buffer systems, PX-E was not used for the production of antiserum.

PX-S(A) formed single precipitin lines with antibodies to PX-S(a) in double diffusion (Fig. 8, pattern 1) and in immunoelectrophoretic (Fig. 9, pattern 1) experiments. PX-E(B) gave a single precipitin line with antibodies to PX-S(a) as well as a line of complete fusion with PX-S(A) (Fig. 8, pattern 2). The

PX-S(A) and PX-E(B) displayed very similar mobilities on agar immunoelectrophoresis (Fig. 9, pattern 1). A single identical immunoprecipitin line of PX-S(A) and PX-E(B) was obtained also with antibodies to intact endotoxin LPS-U-S(k) (Fig. 8, pattern 5). Immunoelectrophoretic patterns of the endotoxin LPS-U-S(K) with antibodies to LPS-U-S(k) revealed three precipitin arcs, one of which seemed to be identical with the line formed by PX-S(A) (Fig. 9, pattern 2). This line failed to form upon absorption of anti-LPS-U-S serum with PX-S (Fig. 10). The band migrating most rapidly towards the cathode was identified as the polysaccharide side chain (L) of the endotoxin complex (Fig. 9, pattern 3). The third line of LPS-U-S, closest to the antigen well in immunoelectrophoretic patterns (Fig. 9, patterns 2 and 3; Fig. 10), represents most probably the intact endotoxin complex. The presence of three separate lines in LPS-U-S indicates that the endotoxin complex isolated by trichloroacetic acid extraction contained some free polysaccharide side chain and protein moiety. On the other

hand, the immunological characterization of PX-S showed that purified "simple" protein was contaminated neither with the polysaccharide moiety nor with the intact endotoxin complex.

PX-S and PX-E exhibited single, identical lines against antiserum prepared with whole cells of S. marcescens 08. The "simple" proteins and their oxidized derivatives formed lines of complete fusion with antibodies to PX-S(a) (Fig. 8, pattern 2), to PXOD-S(c) (Fig. 8, pattern 3), to PXOD-E (Fig. 8, pattern 4), and to LPS-U-S(k) (Fig. 8, pattern 5). These results indicated that the oxidized proteins retained immunological identity with the parent protein preparations PX-S and PX-E, and that antisera developed with endotoxin or "simple" protein preparations from S. marcescens 08 cross-reacted with corresponding fractions from E. coli and vice versa.

#### DISCUSSION

Results of this study have shown that the "simple" proteins isolated by aqueous phenol treatment (17) of either the whole

endotoxin complex of S. marcescens 08 or intact cells of E. coli O 141:K85(B) contain also the characteristic constituents of lipid A such as glucosamine, phosphorus,  $\beta$ -hydroxy myristic and other fatty acids. Although lipid A or its degradation fragments could occur as contaminations in the protein preparations, we suggest that lipid A constituents are firmly bound as an entity to the protein moiety. This conclusion is based on the following evidence: 1) contamination by lipopolysaccharides can be excluded, since hydrolysis of "simple" proteins revealed the presence of no other sugars except glucosamine; furthermore, the phenol phases were washed with water until no immunologically-detectable lipopolysaccharides could be identified in two successive washings, 2) a similar contamination by lipid A can also be eliminated, since exhaustive extraction of protein preparations by chloroform removed free fatty acids, glycerides and phospholipids, but not lipid A or its characteristic constituents, and 3) the protein preparations PX-S and PX-E and their oxidation products PXOD-S and PXOD-E were

homogeneous on the basis of analytical ultracentrifugation, agarose electrophoresis, immunodiffusion and immunoelectrophoresis. Thus, contrary to earlier views (3-7), the protein moieties of endotoxins isolated by aqueous phenol cannot be classified as "simple" proteins if this term implies absence of constituents other than amino acids.

Although Morgan and Partridge (3-5) and Goebel et al. (6, 7) realized that endotoxins contained a component other than protein and polysaccharide, it remained for Westphal and Lüderitz (1) to initiate a systematic investigation into the nature of the lipid moiety, to recognize the principle components of lipid A, and to suggest that the so-called toxic "factor T" (6, 7) and "prosthetic group" (3-5) were probably identical with lipid A. Before the composition of lipid A became known, the absence or a low content of phosphorus had been used as the exclusive criterion for differentiation between "simple" and "conjugated" protein. The failure to detect glucosamine (7) in "simple" and "conjugated"

toxic protein of Shigella paradysenteriae might have been the reason why later investigators did not search at all for glucosamine in the protein moieties of endotoxins; the  $\beta$ -hydroxy myristic acid was not recognized as a specific marker for lipid A until 1953 (42). These facts, and possibly the lack of sensitive analytical methods, may explain why lipid A was not recognized as an integral part of "simple" protein.

Morgan and Partridge (4) recognized the "simple" and "conjugated" proteins of Salmonella typhosa and Shigella dysenteriae as weak antigens. The "simple" and "conjugated" proteins of Shigella paradysenteriae (7) produced antibodies which reacted with the intact O-antigenic complex as well as with the homologous antigen. Results of our study not only confirmed the immunogenic properties of "simple" protein from S. marcescens but revealed also the presence of a common antigenic determinant in "simple" proteins of both S. marcescens and E. coli. The oxidation of PX-S and PX-E by performic acid did not destroy the antigenic site, since both PXOD-S and PXOD-E were still immunogenic.



The similarity in the chemical composition and the presence of a common antigenic determinant in the endotoxin proteins from S. marcescens and E. coli represent the first experimental evidence for a recent suggestion (2) that protein components of many Gram-negative bacteria are similar.

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Table 1  
Elementary Analysis and Chemical Composition of PX-S, PX-E, PXOD-S and PXOD-E

	C	H	N	S	PO <sub>4</sub>	Anthrone- positive carbohydrates	Glucosamine	Fatty Acids	Amino Acids	Recovery <sup>a</sup>	Ash
	%	%	%	%	%	%	%	%	%	%	%
PX-S	51.23	6.92	13.43	0.32	2.01	1.9	2.9	10.1	72.9	89.8	4.9
PX-E	48.44	7.66	15.18	1.03	1.59	1.2	1.3	6.0	83.2	93.3	3.2
PXOD-S	45.10	6.26	10.94	0.74	1.44	1.8	2.6	6.6	66.2	78.6	7.0
PXOD-E	43.31	6.31	14.19	1.24	1.17	1.1	1.1	3.7	72.6	79.7	6.0

<sup>a</sup>Recovery was calculated from anthrone-positive carbohydrates, PO<sub>4</sub>, glucosamine, fatty acids and amino

acids.

Table 2  
Percent Fatty Acid Composition of PX-S, PX-E,  
PXOD-S and PXOD-E

	PX-S %	PX-E %	PXOD-S %	PXOD-E %
C <sub>12</sub>	2.6	3.85	5.23	-
C <sub>14</sub>	9.5	5.52	5.23	14.0
C <sub>14-1</sub>	Trace	-	2.53	-
Unknown	12.9	-	12.75	-
C <sub>16</sub>	16.5	26.42	10.46	30.0
C <sub>16-1</sub>	5.5	-	-	-
C <sub>18</sub>	-	38.79	-	10.0
C <sub>18-1</sub>	Trace	4.34	-	15.0
Unknown	Trace	-	-	-
Unknown	3.9	-	-	-
Unknown	4.5	-	-	-
C <sub>14-OH</sub>	44.5	15.88	63.79	29.0



Table 3

Amino Acid Composition of PX-S, PX-E, PXOD-S, and PXOD-E

	PX-S		PX-E		PXOD-S		PXOD-E	
	$\mu\text{Moles/g}$	mg/100	$\mu\text{Moles/g}$	mg/100	$\mu\text{Moles/g}$	mg/100	$\mu\text{Moles/g}$	mg/100
Lysine	245	3.58	234	3.42	160	2.41	232	3.39
Histidine	74	1.15	87	1.34	48	0.78	62	0.96
Arginine	181	3.15	212	3.65	151	2.61	242	4.22
Aspartic acid	824	10.97	786	10.42	813	12.39	656	8.73
Threonine	422	5.03	384	4.57	384	5.21	295	3.51
Serine	442	4.64	354	3.71	385	4.48	319	3.35
Glutamic acid	537	7.90	910	13.32	479	8.02	879	12.93
Proline	137	1.58	253	2.91	100	1.29	198	2.28
Glycine	657	4.93	653	4.90	607	5.29	466	3.50
Alanine	657	5.85	798	7.10	635	6.54	964	8.59
1/2 Cystine	-	-	-	-	-	-	-	-
Valine	378	4.43	454	5.31	344	4.39	423	4.97
Methionine	42	0.63	145	2.16	88 <sup>a</sup>	1.72 <sup>a</sup>	137 <sup>a</sup>	2.04 <sup>a</sup>
Isoleucine	238	3.12	329	4.30	182	2.49	298	3.91
Leucine	478	6.27	623	8.16	375	5.71	527	6.91
Tyrosine	240	4.35	209	3.79	Traces	Traces	Traces	Traces
Phenylalanine	228	3.77	220	3.62	175	3.29	203	3.35
Tryptophan	75	1.54	27	0.55	-	-	-	-

<sup>a</sup> Methionine sulfoxide

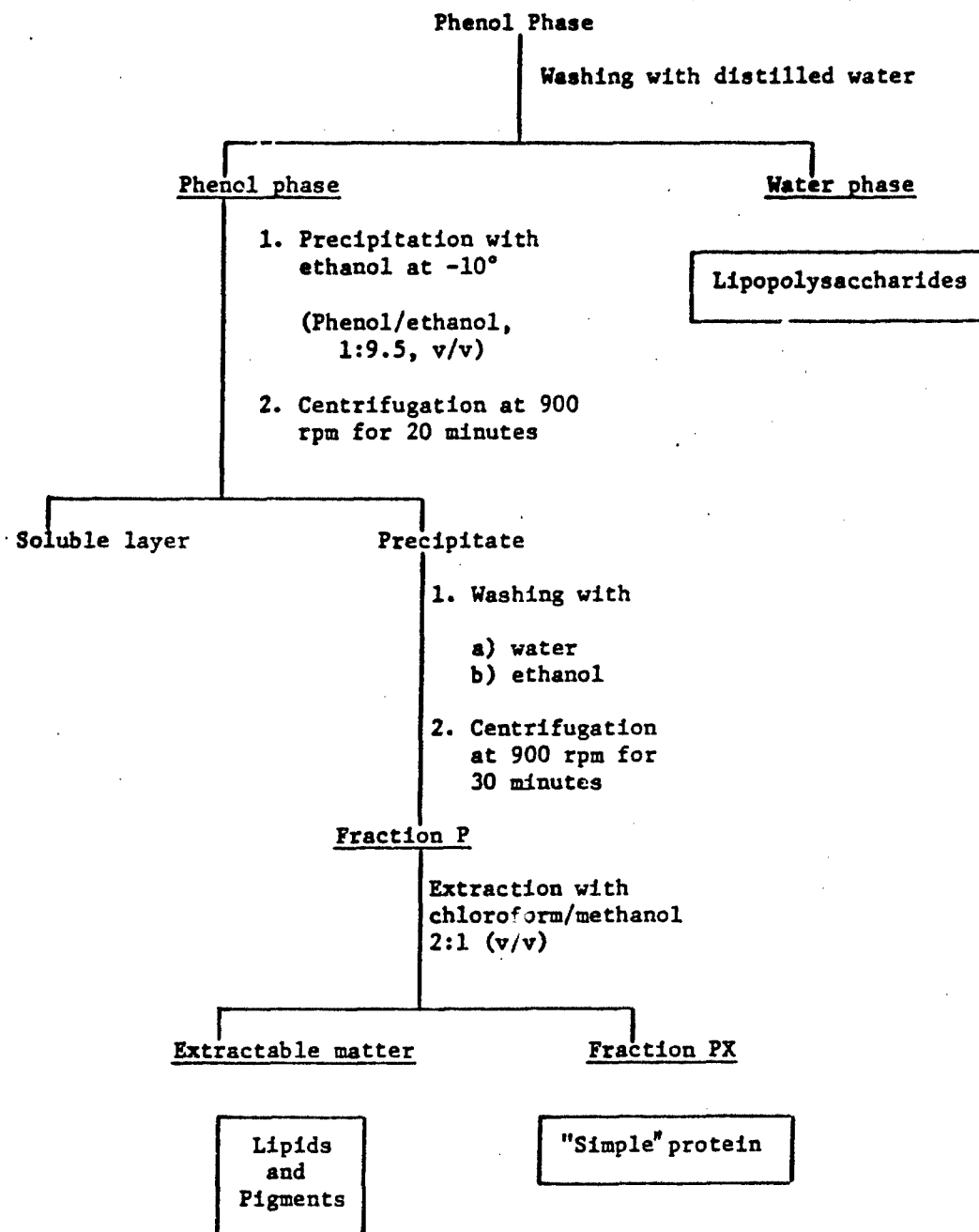


Figure 1. Isolation of "simple" proteins.

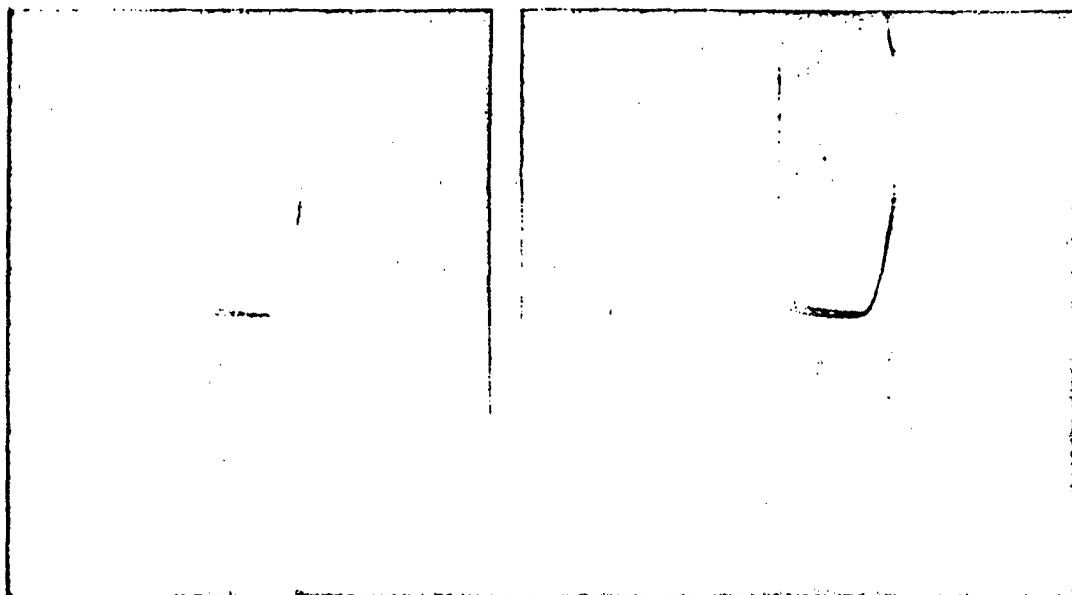


Figure 2. Sedimentation velocity pattern of PX-S. PX-S (5 mg/ml) dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.2% SDS, solvent density 1.004 g/ml, was centrifuged in a synthetic boundary cell at 52,640 rpm at 25°. Exposures were taken from left to right at 4 minute intervals for a total run of 64 minutes. Photographs shown were obtained at 12 and 32 minutes after reaching speed.

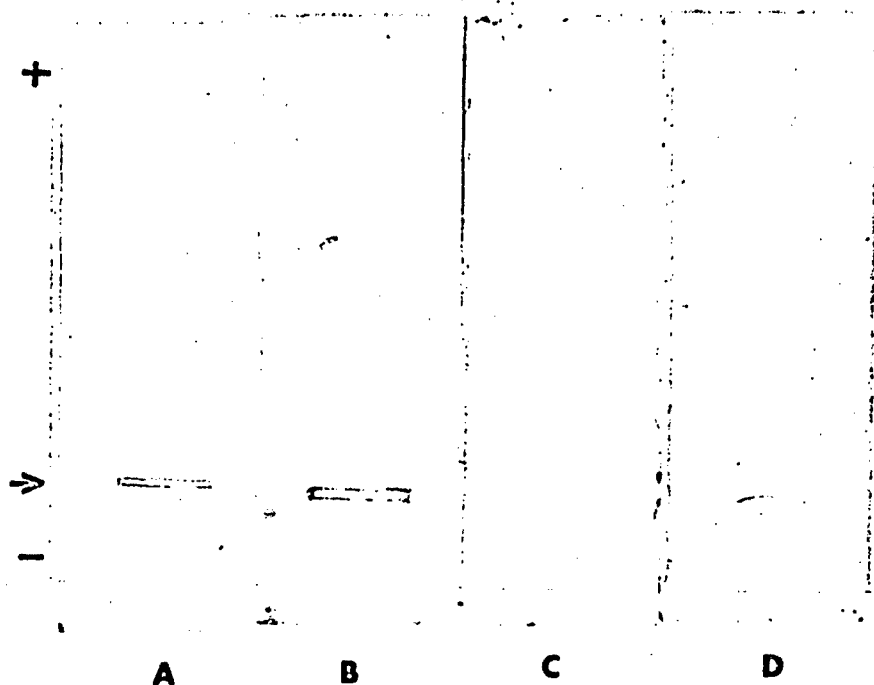


Figure 3. Agarose electrophoresis of PX-S(A), PX-E(B), PXOD-S(C) and PXOD-F(D). Protein preparations PX-S and PX-F (20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in Veronal buffer. Protein preparations PXOD-S and PXOD-E (20 mg/ml) were dissolved in borate buffer, pH 8.9, and diluted to a final concentration of 10 mg/ml with 1% agarose. Electrophoresis was performed in 1% agarose gel using Veronal buffer, pH 8.6, ionic strength 0.05 for 40 minutes.

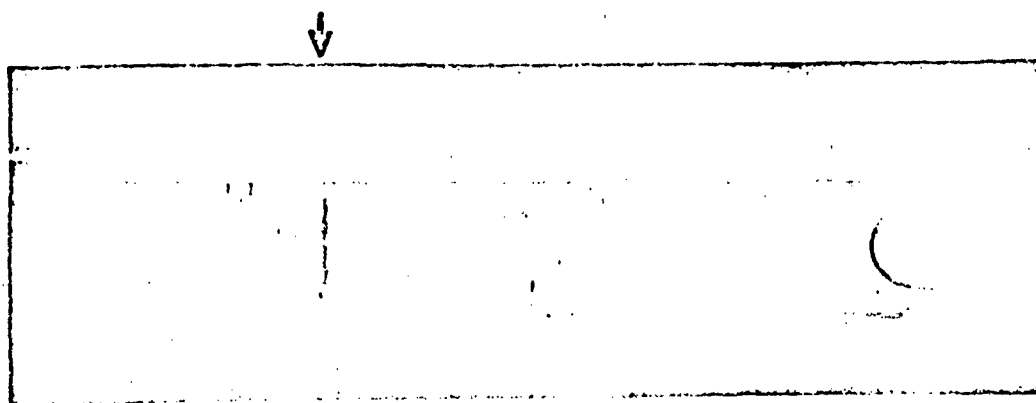


Figure 4. Polyacrylamide gel electrophoresis of PX-S. PX-S (10 mg/ml) was dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.5 SDS. Electrophoresis was carried out in 7.5% polyacrylamide gel employing continuous buffer system Tris-glycine, pH 8.8. Arrow indicates the purple colored band of PX-S. The color is due to the firmly attached prodigiosin; the same band was stained also with Amido Black 10B.

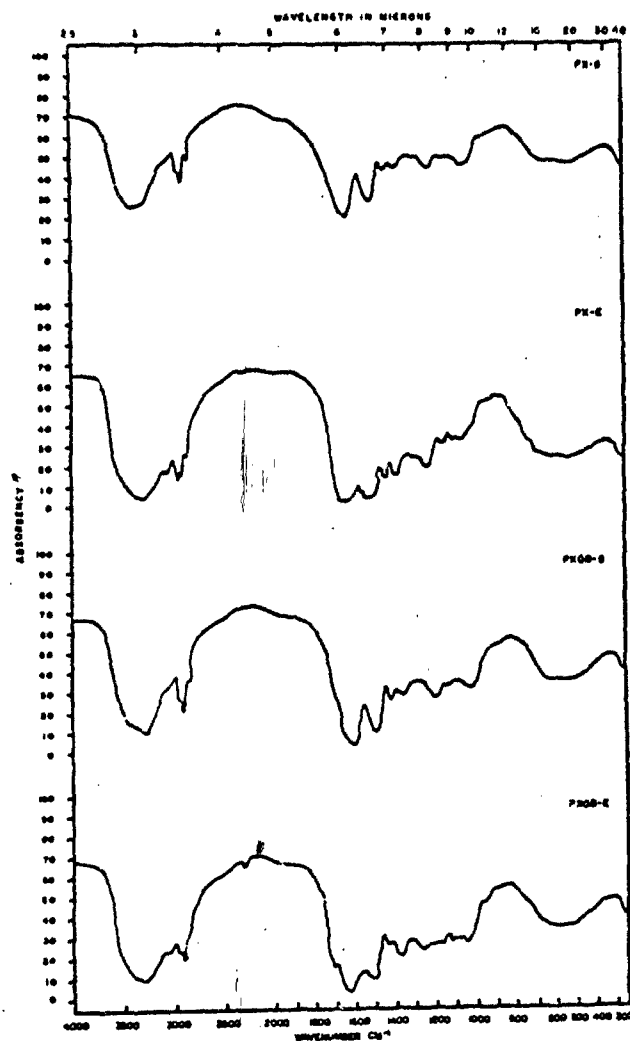


Figure 5. Solid infrared spectra of PX-S, PX-E, PXOD-S, and PXOD-E.

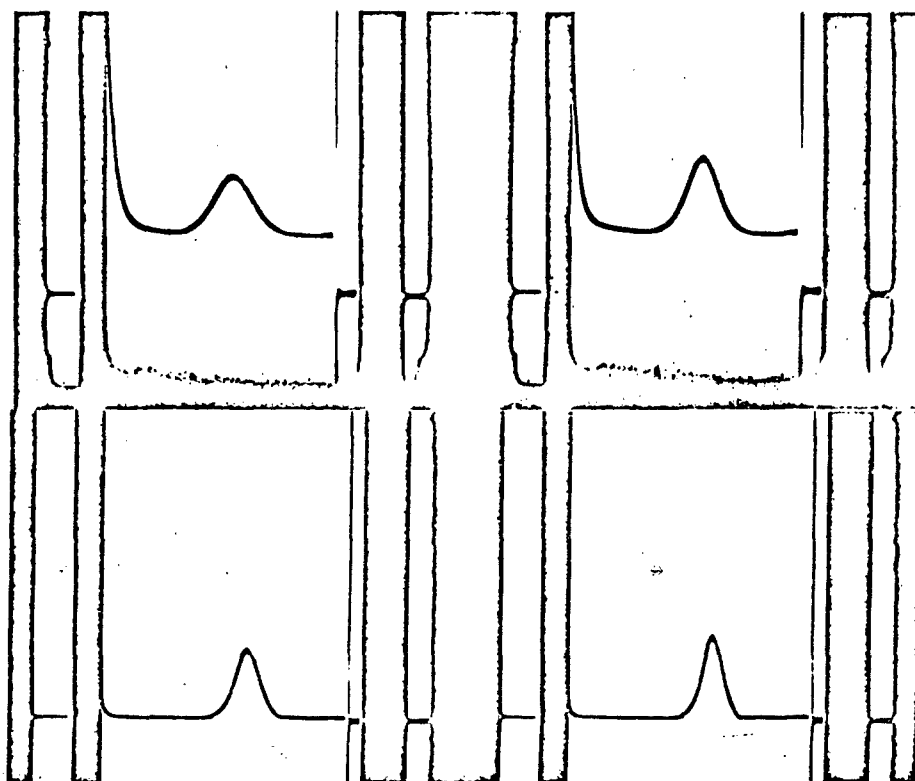


Figure 6. Sedimentation velocity patterns of PXOD-S (top) and PXOD-F (bottom). Protein preparations (10 mg/ml) dissolved in borate buffer, pH 8.9, solvent density 1.004 g/ml, were centrifuged in synthetic boundary cells for 64 minutes at 52,640 rpm at 25°. Photographs were taken from left to right at 12 and 32 minutes after reaching speed.

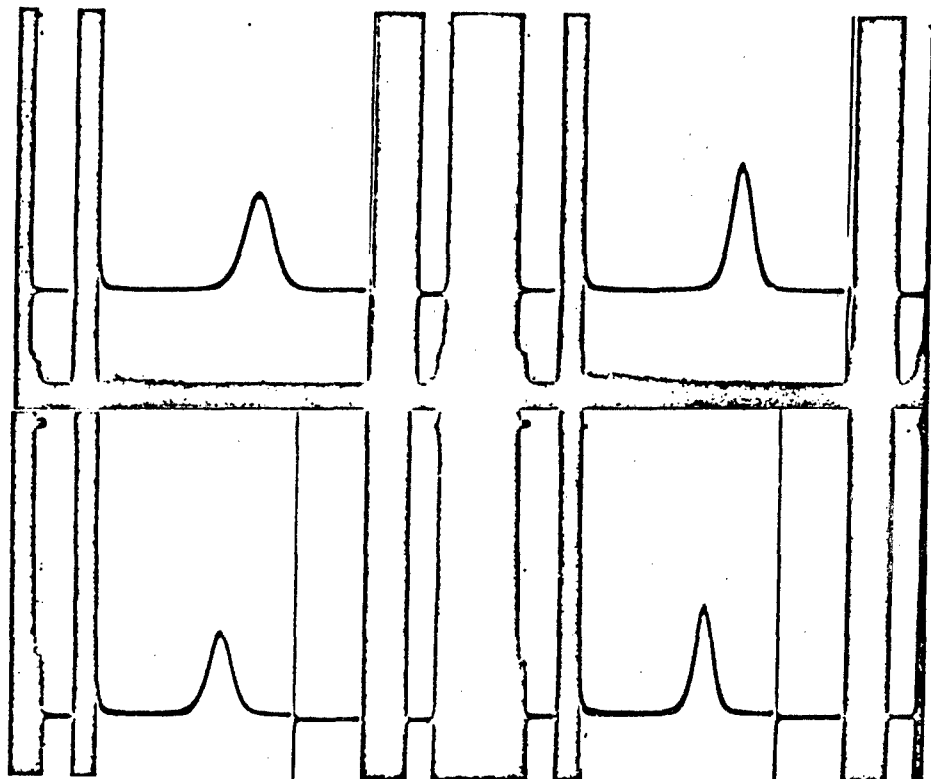


Figure 7. Diffusion patterns of PXOD-S (top) and PXOD-E (bottom). Prote preparations (10 mg/ml) dissolved in borate buffer, pH 8.0, density 1.004 g/ml, were centrifuged in synthetic boundary cell for 64 minutes at 12,590 rpm and 25°. Photographs taken from left to right were obtained at 12 and 32 minutes after reaching spe



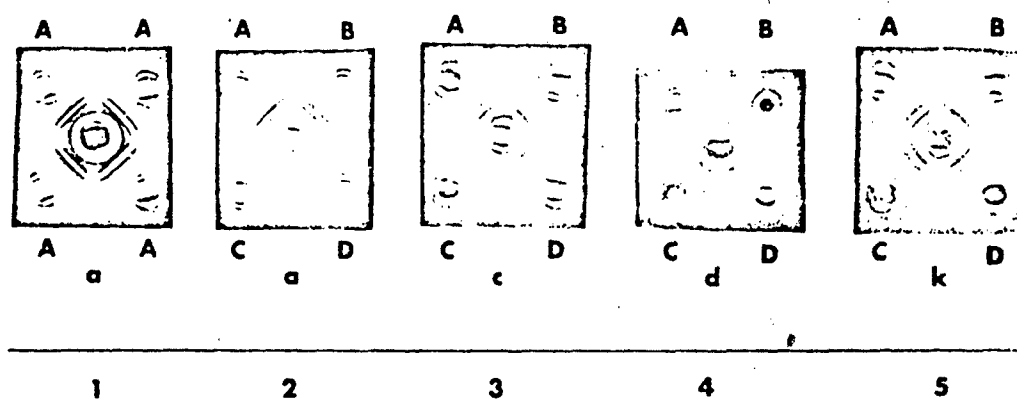


Figure 8. Immunodiffusion patterns of PX-S(A), PX-E(B), PXOD-S(C), and PXOD-E(D). Central wells contain antibodies to PX-S(a), PXOD-S(c), PXOD-E(d), and LPS-U-S(k).

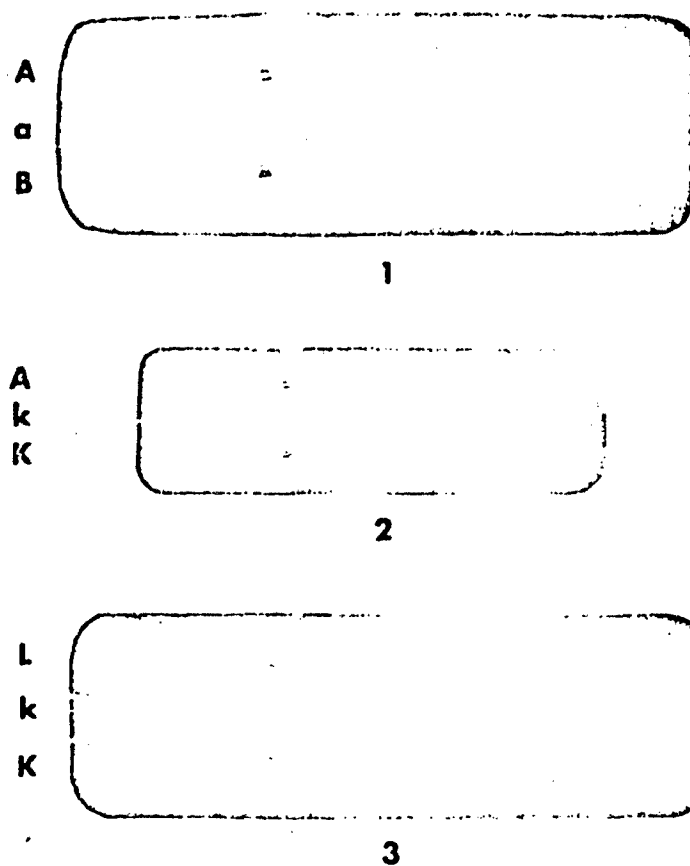


Figure 9. Immunoelectrophoretic patterns of PX-S(A), PX-E(B), LPS-U-S(K) and the polysaccharide side chain (L). Central trough in pattern 1 contains antibodies to PX-S(a) and the central troughs in patterns 2 and 3 contain antibodies to LPS-U-S(k).

A  
k  
K

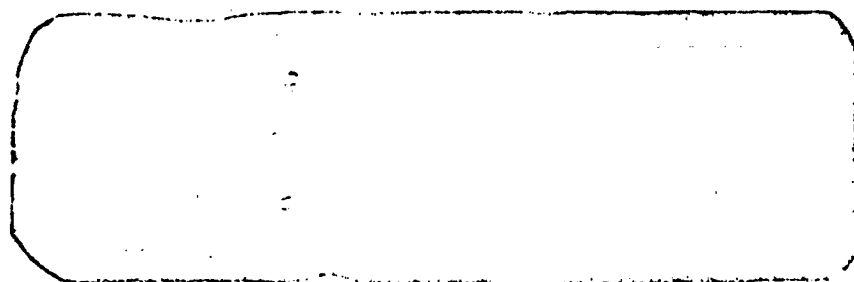


Figure 10. Immunoelectrophoretic pattern of PX-S(A) and LPS-U-S(K).  
Central trough contains anti LPS-U-S serum absorbed with  
PX-S(A).

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13. ABSTRACT The so-called "simple" proteins isolated from the endotoxin of <u>S. marcescens</u> 08 and whole cells of <u>E. coli</u> 0 141:K85 (B) by aqueous phenol treatment were charac- terized by the determination of hydrodynamic properties, electrophoretic behavior, immunochemical specificity and chemical analysis. The chemical composition of both proteins revealed the presence of lipid A constituents such as glucosamine, phos- phorus and fatty acids; the B-hydroxy myristic acid regarded as the characteristic marker for lipid A was present in all protein preparations. Evidence presented in this paper indicates that a portion of lipid A is firmly bound to the protein moieties. Therefore, endotoxin protein preparations isolated by aqueous phenol cannot be classified as "simple" proteins if this term implies absence of compounds other than amino acids. The protein moieties from <u>S. marcescens</u> and <u>E. coli</u> were immunogenic and possessed a common antigenic determinant.			

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